Effect of Vitamin E on Lipid Peroxidation and Fertility After Artificial Insemination with Liquid-Stored Turkey Semen

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ABSTRACT Turkey sperm plasma membranes contain high levels of polyunsaturated fatty acids that are susceptible to lipid peroxidation during in vitro storage at 4°C. Herein we assessed the degree of lipid peroxidation and fertility potential of semen liquid-stored for 24 h with the antioxidant vitamin E. Semen was collected weekly from 44 males and pooled as pairs (total = 22); the individuals in paired samples exhibited similar semen quality parameters. After initial semen evaluation, pooled samples were extended with Beltsville Poultry Semen Extender containing no supplement (control) or 10 or 40 μg/mL vitamin E and then stored at 4°C with constant aeration for 24 h. Lipid peroxidation was determined by measuring malonaldehyde (MDA) in aliquots (50×10^6 sperm) of fresh (0 h) and stored (24 h) semen. Sperm mobility was also evaluated. A total of 176 hens (8 hens/tom pair; 4 hens/0 h, 4 hens/24 h) were inseminated (150×10^6 sperm) weekly for 6 wk, and fertility was determined after 7 d of incubation. Initial MDA values of the 22 tom pairs ranged from 0.928 to 1.36 u*M*. Males varied in production of MDA during in vitro storage, with most pairs exhibiting a threefold increase. Results indicated that supplemental vitamin E did not reduce lipid peroxidation during liquid storage. Not surprisingly, artificial insemination with stored semen (with much higher MDA values) yielded lower fertility rates than control regardless of the presence of vitamin E. These results demonstrate that lipid peroxidation is a significant factor affecting the fertility of stored turkey sperm and that methods to prevent or reduce lipid peroxidation remain to be elucidated.

(*Key words*: turkey, semen, liquid storage, lipid peroxidation, vitamin E)

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INTRODUCTION

Despite heavy dependence of the turkey industry on artificial insemination (AI) for production, successful methods for long-term in vitro storage of turkey semen have not been developed. Undiluted turkey semen does not have the capacity to remain viable for long periods outside the reproductive tract (Leighton et al., 1968). Furthermore, diluted turkey semen becomes anaerobic within 15 min at 10°C (Wishart, 1981). It is well established that turkey spermatozoa provide energy for metabolic requirements by oxidative respiration rather than glycolysis (Sexton, 1974; Wishart, 1981; Sexton and Giesen, 1982) and thus benefit from a supply of molecular oxygen during in vitro storage. Although the fertilizing capacity of turkey semen is improved by continuous introduction of air during storage, fertility rates of turkey semen stored for longer than 6 to 12 h (38 to 74%) are still far below what the industry requires (96 to 98%).

The paradox of providing sufficient oxygen for sperm metabolism during liquid storage is that molecular oxygen also is involved in degradation of lipids through peroxidation, and lipids are major components of avian sperm. For example, almost 60% of the total sperm lipids in turkey sperm cells are phospholipids (Cerolini et al., 1997). Avian sperm phospholipids are further characterized by a large proportion of polyunsaturated fatty acids (PUFA) that are associated with a high peroxidizability index (Surai et al., 2001).

Physiologically, the high proportions of PUFA in avian sperm are integral for maintaining membrane fluidity and flexibility during the fertilization process. Numerous studies have demonstrated a direct link between compromised poultry sperm function after in vitro storage and lipid peroxidation (Fujihara and Howarth, 1978; Fugihara and Koga, 1984; Wishart, 1984; Cecil and Bakst, 1993). Damaging effects of lipid peroxidation on poultry sperm

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Abbreviation Key: AI = artificial insemination; BPSE = Beltsville Poultry Semen Extender; MDA = malonaldehyde; PUFA = polyunsaturated fatty acid; TBA = thiobarbituric acid; VE = vitamin E.

include morphological defects, reduced motility, and poor fertilizing ability. Research with mammalian spermatozoa has documented other deleterious effects of lipid peroxidation, such as loss of membrane fluidity (Borst et al., 2000), decreased acrosomal function (Jones and Mann, 1977; Ford and Williams, 2001), damage to sperm chromatin (Kodama et al., 1997), and reduced sperm-oocyte fusion (Kodama et al., 1996), which also may be involved in the compromised fertility of stored poultry semen.

Although turkey semen loses acceptable fertility after only a few hours of in vitro storage at 5°C, sperm maintained for 6 to 8 wk at body temperature in the turkey hen sperm storage tubules are still capable of fertilization (Wishart, 1989). Given the susceptibility of turkey sperm PUFA to lipid peroxidation, an efficient antioxidant system would be required in the semen or the hen's sperm storage tubules to protect sperm membranes from peroxidative damage (Kelso et al., 1996). The antioxidant vitamin E is present in turkey semen, with a higher concentration localized within sperm cells than in seminal plasma (Surai, 1981). Vitamin E is a natural stabilizer of poultry sperm plasma and mitochondrial membranes (Surai and Ionov, 1992) and has been demonstrated to improve turkey sperm mobility and viability during storage for 48 h at 5°C when incorporated into the extender (Donoghue and Donoghue, 1997). This experiment was designed with several objectives: (1) to evaluate the degree of lipid peroxidation occurring during liquid storage using a novel colormetric malonaldehyde assay, (2) to evaluate the ability of vitamin E to reduce lipid peroxidation, (3) to examine the peroxidizability index of semen and other characteristics of individual toms in relation to fertility, and (4) determine whether the fertility of turkey semen would be enhanced by the addition of vitamin E during in vitro storage.

MATERIALS AND METHODS

Turkey Management

Large White breeder poults were purchased from a primary breeder[2] and maintained under standard management conditions during the brooding and growing periods. At 28 wk of age, toms were photostimulated by increasing light exposure from a 12L:12D to a 14L:10D cycle to initiate semen production. By using the abdominal massage semen collection method (Burrows and Quinn, 1935), toms were premilked at 30 wk of age and then screened for semen quality at 31 and 32 wk of age prior to being designated to sperm storage treatment groups. At 32 wk of age, hens were photostimulated by increasing the daily light exposure from 6L:18D to 14L:10D to initiate ovarian development and egg production. Hens were inseminated on d 10 and 12 after photostimulation and then once per week for the next 14 wk of egg production.

Vitamin E Supplementation and Liquid Storage of Semen

Semen was collected from 44 toms once weekly for 6 wk and evaluated for volume, sperm concentration, pH, osmolarity, sperm viability, and sperm mobility. Raw semen was then aliquoted and diluted 1:1 with nonsupplemented Beltsville Poultry Semen Extender (BPSE; control) or BPSE supplemented with 10 or 40 μg vitamin E/mL. After dilution, semen samples were transferred to 48-well culture plates. Multiwell plates were placed in a room-temperature water bath on an orbital shaker (125 rpm) and slowly cooled to 4°C, with lids loosely fitted to ensure air availability during agitation.

Lipid Peroxidation Assay

Aliquots of fresh (0 h) and stored (24 h) semen from the control and 10 μg and 40 μg vitamin E BPSE treatments containing 50×10^6 sperm/mL were frozen at –70°C prior to being analyzed for lipid peroxidation. Lipid peroxidation in fresh and stored semen was evaluated using a modified colormetric assay[3] to detect malonaldehyde (MDA) concentration. MDA, an end-product of lipid peroxidation, reacts with the chromogenic agent N-methyl-2^2-phenylindole to form a stable chromophore with maximal absorbance at 586 nm. The reaction time and temperature were increased to 80 min and 60°C, respectively, to measure free and protein-bound MDA in the semen samples. Further modifications of the MDA-586 assay included using 96-well plates to conduct the colormetric reaction and a plate reader to measure absorbance. The MDA concentrations of semen samples were calculated from standard curves generated from known quantities of MDA (0, 0.5, 1.0, 2.0, 3.0, 4.0 μM).

Sperm Mobility and Viability Assays

Sperm mobility was assessed according to the methods of Donoghue et al. (1998), except that the sperm suspension (1×10^9 sperm/mL mobility buffer) was overlaid onto a 6% Accudenz[4] solution (King et al., 2000). The optical density was measured by a microreader photometer.[5] Sperm viability was determined using the SYBR-14/propridium iodide live per dead stain combination as evaluated by flow cytometry (Donoghue et al., 1995).

Fertility Evaluation

A total of 176 hens were artificially inseminated weekly with fresh (n = 88) or stored (n = 88) semen extended with control (n = 30, fresh; n = 30, stored), 10 μg vitamin E (n = 28, fresh; n = 28, stored), or 40 μg vitamin E (n = 30, fresh; n = 30, stored) BPSE. The inseminant dose for all treatments was 150×10^6 sperm. Lower sperm numbers

[2]British United Turkeys of America, Lewisburg, WV.
[3]MDA-586, Oxis Research, Portland OR.
[4]Accudenz, Accurate Chemical & Scientific Corp., Westburg, NY.
[5]Microreader photometer, IMV, Minneapolis, MN.

than typically inseminated for commercial production were used because we desired a system sufficiently sensitive to detect even small changes in fertility due to sperm treatment. Eggs were candled after 7 d of incubation to determine true fertility.

Experimental Design

Prestudy screening was used to assign individual males with similar semen quality traits (semen volume, sperm concentration, sperm viability, and sperm mobility) to a storage treatment consisting of two males per pool for a total of 22 semen pools. Semen was pooled in this manner throughout the study to (1) minimize variability associated with semen pools of multiple (5 to 6) males and (2) provide a mechanism for evaluating the peroxidizability index of semen and other semen qualities from each male pair. Because the semen sample volumes limited allocation of each pool to all three storage treatments, pooled semen samples were randomly assigned to two extender treatments in an incomplete block design. Semen from seven of the 22 pools was split into two aliquots and diluted with either control or 10 μg vitamin E BPSE. Similarly, semen from eight other pairs was allocated to control and 40 μg vitamin E BPSE. Semen from the remaining seven pairs was stored in the presence of 10 or 40 μg vitamin E BPSE. This experimental design resulted in 15, 14, and 15 paired semen samples represented in the control, 10, and 40 μg vitamin E BPSE treatments, respectively.

Statistical Analysis

Prior to analysis, data were checked to determine if a transformation was necessary. As expected, variables scored as percentages (mobility, viability, and fertility) required transformation. We used the standard variance stabilizing arcsine transformation, y = arcsine ($\sqrt{(x)}$), where x = data, recorded as a proportion; and y = scale used in the analysis. This transformation stabilized the variances sufficiently to proceed with an analysis in a mixed models framework. Software for conducting mixed models analyses in non-Gaussian frameworks, e.g., logistic regression, is not yet readily available. MDA concentration did not require transformation. The mixed models we developed, estimated using Proc Mixed (SAS Institute, 1999), considered sperm treatment and week of study as fixed factors and tom pair and hen (nested in tom pair) as random factors. In addition, because data were taken from the same individuals repeatedly, thus inducing correlation, we allowed for within-individual time series correlation using an autoregressive (1) structure. We also tested MDA concentration, mobility, and viability as potential covariates (predictors) of fertility with and without tom pairs in the model. To ensure that our sample size and design had sufficient power to accurately determine the effects of the sperm treatments, we did a small simulation study prior to finalizing the design. Power was reassessed once the study was complete, and we report those results below. We used the default REML estimation method in Proc Mixed. For modeling fertility, we assumed the variance on the transformed scale due to sampling eggs was 0.25 per n, where n is the number of eggs per hen per week (Steel and Torrie, 1960).

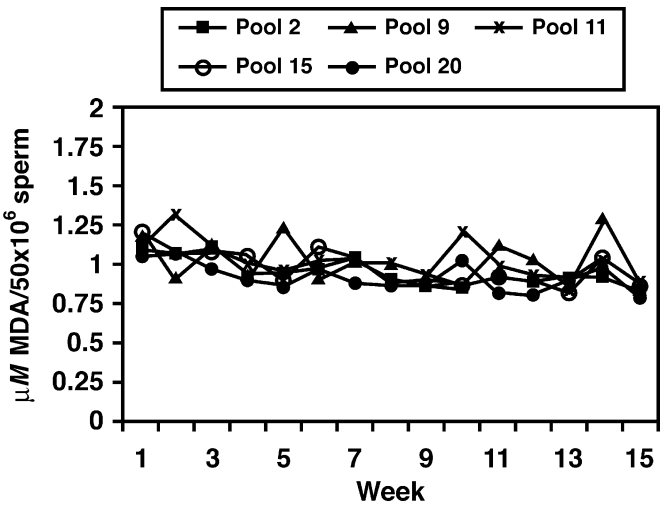

FIGURE 1. Baseline lipid peroxidation in fresh, undiluted turkey semen. Represented in the graph are weekly semen samples from 5/22 tom pairs during the 15-wk production period. Baseline malonaldehyde (MDA) values were similar ($P > 0.05$) for all 22 tom pairs.

RESULTS

The MDA was detected in fresh ejaculates from all toms throughout the 15-wk production period (Figure 1); however, the concentrations were low, ranging from 0.7 to 1.2 μM/50 $\times$ 10^6 sperm. After 24 h of storage with constant aeration, MDA levels were threefold higher than that detected in fresh semen (Figure 2). Additionally, semen from eight of the 44 toms consistently contained high MDA concentrations (>4.0 $\mu M/50 \times 10^6$ sperm) after liquid storage without vitamin E. Interestingly, the baseline levels of MDA in fresh ejaculates from these eight males were similar to the remaining toms ($P > 0.05$).

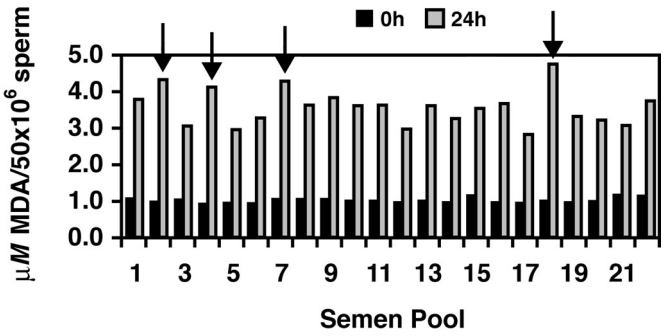

FIGURE 2. Lipid peroxidation in fresh and stored turkey semen. Represented are the average malonaldehyde (MDA) values of each tom pair during the production period (n = 15 samples per tom pair) at 0 and 24 h of storage. Baseline MDA values (0 h) did not predict which tom pairs would produce high levels of MDA (indicated by arrows) after in vitro storage (24 h).

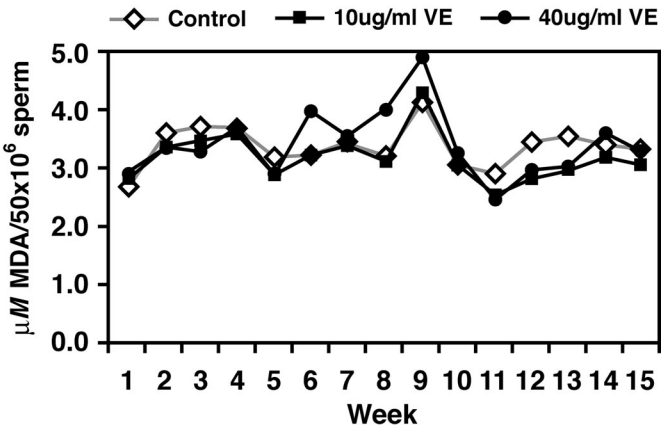

FIGURE 3. Effect of vitamin E (VE) supplementation on lipid peroxidation of stored turkey semen. Represented are average malonaldehyde (MDA) values of semen stored for 24 h with or without VE (control, n = 15; 10 μg/mL VE, n = 14; 40 μg/mL, n = 15). There were no significant differences ($P > 0.05$) in weekly MDA values between control and VE treatments.

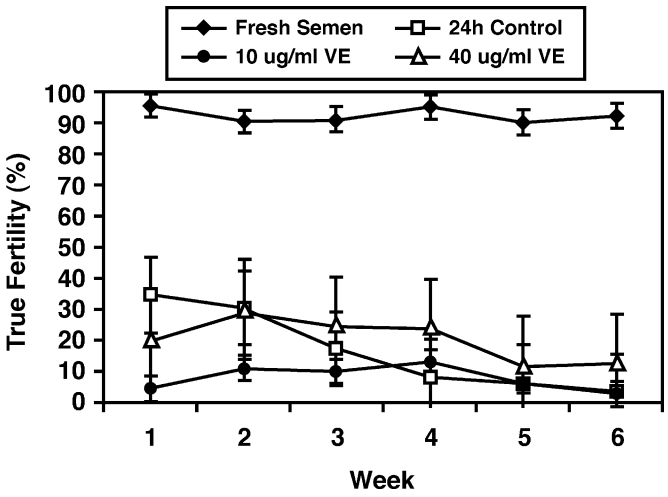

FIGURE 5. Fertility of fresh and stored turkey semen with or without vitamin E (VE). Fertility rates were similar ($P > 0.05$) between control and both VE treatments for fresh semen, therefore 0 h fertility data were pooled. Fertility rates of stored semen were consistently lower ($P < 0.05$) than fresh semen. For stored semen fertility rates, no significant differences ($P > 0.05$) were detected between control and VE treatments.

Addition of 10 or 40 μg/mL vitamin E to the storage extender did not consistently reduce ($P > 0.05$) the levels of MDA in stored semen samples (Figure 3). In particular, weekly MDA concentrations in the semen samples stored with 40 μg/mL vitamin E varied widely (2.4 to 4.8 μM/ 50×10^6 sperm) throughout the study. Although semen stored with vitamin E appeared to contain lower levels of MDA than control semen during wk 11 to 15 of the study (Figure 3), the differences were not significant ($P > 0.05$).

Sperm mobility was consistently lower ($P < 0.05$) after 24 h of semen storage than in fresh samples, regardless of semen treatment (Figure 4). Although sperm viability was not improved ($P > 0.05$) by the presence of vitamin E during liquid storage of semen, sperm viability was not different after 24 h of storage for any of the semen treatments. For fresh semen, the percentage of live sperm ranged from 86.2 to 87.7% in the control and two vitamin E treatments. Similarly, percentages of live sperm in stored control and vitamin E treatments ranged from 79.1 to 81.4%. Although there was no overall correlation between sperm viability and MDA concentration, impaired sperm mobility in stored semen occurred with higher levels of MDA ($P < 0.05$).

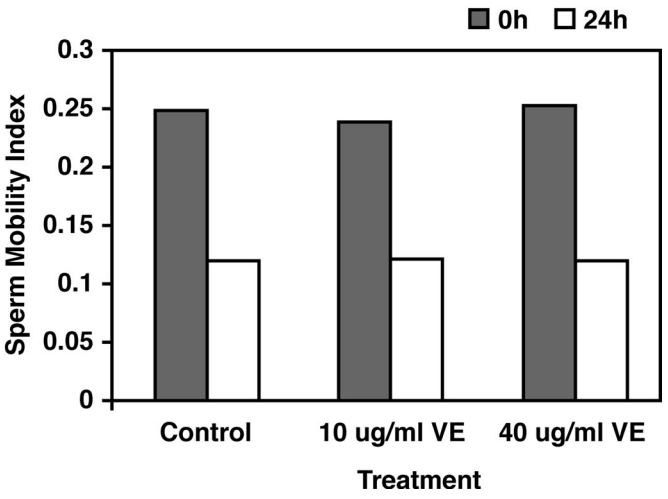

FIGURE 4. Effect of vitamin E (VE) supplementation on sperm mobility of stored turkey semen. Represented are average sperm mobility values for wk 1 to 15 of the study (control, n = 15; 10 μg/mL VE, n = 14; 40 μg/mL, n = 15). Within treatment, 0 h mobility values were consistently higher ($P < 0.05$) than 24-h mobility values. The presence of VE did not improve ($P > 0.05$) sperm mobility in stored semen compared to control.

The fertility rates of fresh semen remained high; however, the average fertility of semen stored for 24 h did not exceed 35% during the study. The overall fertility of stored semen was not improved by addition of vitamin E to the storage extender (Figure 5). Semen that was stored 24 h with 10 μg/mL vitamin E did not produce higher fertility rates than controls ($P > 0.05$); however, the fertility of semen from 2/15 tom pairs stored with 40 μg/mL vitamin E was improved ($P < 0.05$) when compared to respective control or 10 μg/mL vitamin E treatment. Although the semen of a few male pairs significantly improved, there was no relationship between average fertility and whether there was improvement from the 40 μg/mL vitamin E treatment. Other semen characteristics (sperm mobility, sperm viability) measured on tom pairs also did not separate the toms. Note that these results were not due to lack of power to detect the effects of vitamin E treatment. At power = 90% and with our design, an improvement of no more than 0.135 (on the transformed scale, roughly 14% on the original scale) could go undetected.

None of the potential covariates that might predict fertility, MDA concentration, viability, and mobility was significant whether or not tom pair was specified in the model. For fertility, we found the estimated tom pair

effect (variance component) to be 0.017 and the hen effect to be 0.072 (on the transformed scale).

DISCUSSION

Early studies demonstrated that turkey spermatozoa require aerobic conditions for in vitro metabolism (Sexton, 1974; Wishart, 1981) yet oxygenation during liquid storage promotes lipid peroxidation. In this study we demonstrated that some lipid peroxidation naturally occurs within the male reproductive tract and that this low level of peroxidation does not impair the fertilizing ability of turkey semen. Similar work with roosters has shown that peroxides are present at the time of ejaculation (Blesbois et al., 1993); however, the reported MDA values (2 to 4×10^{-2} nM MDA/10^9 sperm) were much lower than the baseline MDA present in turkey ejaculates in this study (14 to 24 μM MDA/10^9 sperm). This apparent discrepancy most likely was the result of the new, more sensitive assay we used in the current study, which measured total (free and protein-bound) MDA by the formation of a stable chromophore. Previous studies with poultry sperm used a thiobarbituric acid (TBA) assay that only measures free MDA and is now known to be less accurate, as other TBA-reactive substances can interfere with the MDA-TBA reaction.

Lipid peroxidation in undiluted poultry ejaculates develops during aerobic incubation in a time-and-temperature-dependent manner. Under anaerobic conditions, rooster semen formed limited amounts of MDA over time, whereas aerobically stored samples produced linearly increasing levels of MDA during a 5-h period (Wishart, 1984). Similarly, aerobic storage for several hours at room temperature increased lipid peroxidation in turkey semen (Cecil and Bakst, 1993). Even at low temperatures (4 to 5°C), lipid peroxidation occurs in chicken (Blesbois et al., 1993) and turkey (Cecil and Bakst, 1993) semen. Furthermore, we have demonstrated that individual toms differ in the degree of lipid peroxide production during liquid storage. Similar data have been reported for rooster semen, in which lipid-peroxide forming capabilities of individual males varies during liquid storage, with some producing 70 times more peroxide than others (Wishart, 1984).

In our study, we purposefully avoided pooling semen from multiple males in order to examine the effect of individual toms on lipid peroxidation. It is interesting that semen from all toms contained baseline MDA levels, with no apparent evidence to indicate which semen samples would produce the largest amounts of toxic peroxides during in vitro storage. Identifying males that characteristically produce high levels of lipid peroxides in vitro would most likely enhance the survivability and fertility of stored turkey semen. For example, when rooster semen from high peroxide males was mixed with low MDA semen samples and stored, the overall lipid peroxide index of the pooled sample was high (Wishart, 1984). Although it seems logical to conclude that semen from high peroxide producers should not be included in pooled semen samples destined for long-term liquid storage, identification of these males would require measurement of MDA after at least 24 h of semen storage, as fresh semen from these males will contain low amounts of MDA.

Natural antioxidants, such as vitamin E, are present in poultry semen. Comparatively lower amounts of vitamin E are present in chicken and drake semen than turkey semen, with very little vitamin E detected in gander semen (Surai and Ionov, 1992). Vitamin E is partitioned between the seminal plasma and spermatozoa, with higher concentrations contained within turkey sperm cells than in the seminal plasma (Surai, 1981). Although naturally present in semen, supplemental vitamin E in semen extenders has yielded inconsistent results in terms of preventing lipid peroxidation. Our data revealed that neither 10 nor 40 μg of supplemental vitamin E was sufficient to limit lipid peroxidation under aerobic storage conditions. Similarly, addition of vitamin E to equine semen did not reduce lipid peroxidation during sperm storage for 48 h at 5°C (Ball and Vo, 2002); however, supplemental vitamin E decreased lipid peroxidation in boar sperm stored at 19°C (Cerolini et al., 2000), and positive effects have been noted for ram sperm stored in vitro (Upreti et al., 1997).

The effects of vitamin E on the mobility and viability of stored sperm also are not consistent. In an earlier study with turkey semen, supplemental vitamin E improved sperm mobility and membrane integrity (Donoghue and Donoghue, 1997), whereas our data do not reflect improvements in either sperm mobility or viability after 24 h in the presence of vitamin E. It is possible that the different experimental designs might have contributed to this discrepancy. In the current study, pairs of toms with similar semen quality were selected and pooled for a larger sample size with less individual male variation, whereas the previous experiment pooled a random group of 20 males. Additionally, the data cannot be directly compared because neither the degree of lipid peroxidation nor fertility rates was examined in the previous study. Beneficial effects of vitamin E (8 μg/mL diluent) on motility and fertility of stored rooster sperm have been reported, although the benefits occurred only early in production for birds 30 to 40 wk old, and lipid peroxidation after storage was not examined (Blesbois et al., 1993).

Work with mammalian species has shown an inverse relationship between degree of lipid peroxidation and sperm mobility in the presence of vitamin E. For example, in the boar, vitamin E improved mobility and decreased lipid peroxidation in stored sperm (Cerolini et al., 2000), whereas equine sperm mobility was not improved and lipid peroxidation was not reduced in the presence of vitamin E (Ball and Vo, 2002). In our study vitamin E provided no benefit for lowering lipid peroxidation or improving sperm mobility of turkey sperm. It is not surprising, then, that the overall fertility of stored turkey sperm was not enhanced by the presence of vitamin E. Lipid peroxidation might have adversely affected sperm energetics that in turn affected the fertilizing ability of

turkey sperm. Further, high MDA concentrations in rooster semen have been associated with a decline in motility and partial or complete loss of fertilizing ability after liquid storage (Fugihara and Koga, 1984; Wishart, 1984). It should also be noted that MDA concentrations are determined from a normalized number of sperm, or 50×10^6 sperm/mL in our study. Therefore, ejaculates containing high sperm concentrations have the potential to produce more MDA during semen storage. Although vitamin E is a natural antioxidant in turkey semen, it seems likely that additional preventative mechanisms, such as increased levels of superoxide dismutase or glutathione, are required to overcome in vitro lipid peroxidation (Froman and Thurston, 1981; Surai et al., 2001). It is clear from our data that addition of vitamin E alone was not sufficient to deter lipid peroxidation during storage of turkey semen.

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